

2011

Human Niemann-Pick Type C2 Disease Protein Expression, Purification and Crystallization

Yurie T. Kim

University of Massachusetts Amherst

Follow this and additional works at: <https://scholarworks.umass.edu/theses>

Kim, Yurie T., "Human Niemann-Pick Type C2 Disease Protein Expression, Purification and Crystallization" (2011). *Masters Theses 1911 - February 2014*. 621.

Retrieved from <https://scholarworks.umass.edu/theses/621>

This thesis is brought to you for free and open access by ScholarWorks@UMass Amherst. It has been accepted for inclusion in Masters Theses 1911 - February 2014 by an authorized administrator of ScholarWorks@UMass Amherst. For more information, please contact scholarworks@library.umass.edu.

HUMAN NIEMANN-PICK TYPE C2 DISEASE PROTEIN EXPRESSION,
PURIFICATION AND CRYSTALLIZATION

A Thesis Presented

by

YURIE T. KIM

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

MASTER OF SCIENCE

May 2011

Molecular and Cellular Biology

HUMAN NIEMANN-PICK TYPE C2 DISEASE PROTEIN EXPRESSION,
PURIFICATION AND CRYSTALLIZATION

A Thesis Presented

by

YURIE T. KIM

Approved as to style and content by:

Scott C. Garman, Chair

Alejandro P. Heuck, Member

Karsten W. Theis, Member

Barbara Osborne, Director
Molecular and Cellular Biology

ACKNOWLEDGMENTS

I would like to give thank to Dr. Scott C. Garman, my principal investigator. For providing opportunity to do the research I love and for continuous support and help even when I was having a hard time and not making too much progress. And because of my language difficulty, he paid special attention to read my work, which was sometime difficult. I give thanks to Nathaniel E. Clark, who taught and guided me from my undergraduate work to the end of my master's degree. Without his guidance and help, I would have not been as well trained as I am now. And I would like to extend my gratitude to other members of Dr. Garman's laboratory for their generous help.

I would like to thank Dr. Alejandro P. Heuck and Dr. Karsten W. Theis for their help to extend my knowledge. I would like to thank Dr. Heuck's laboratory members, especially to Fabian Romano, for helping me use the fluorometer.

During my years in the laboratory, I would have not come this far without help and support from many people, rather I would have given up long before accomplishing what is presented in this dissertation. I give my utmost thanks to those who helped and supported me during the time of my hardship.

ABSTRACT

HUMAN NIEMANN-PICK TYPE C2 DISEASE PROTEIN EXPRESSION, PURIFICATION AND CRYSTALLIZATION

MAY 2011

YURIE T. KIM, B.S., UNIVERSITY OF MASSACHUSETTS AMHERST

M.S., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by: Professor Scott Garman

Niemann-Pick type C2 (NPC2) protein is a soluble protein that binds unesterified cholesterol. The protein helps transport unesterified cholesterol in tandem with the membrane protein Niemann-Pick type C1 (NPC1). Defects in either of proteins can cause Niemann-Pick type C disease (NPC), which results in the accumulation of unesterified cholesterol and lipids in the late endosome and lysosome. NPC is an autosomal recessive lysosomal storage disease affecting 0.35~2.20 per 100,000 people. Here we present the structural analysis of the human NPC2 glycoprotein, including expression, purification, functional analysis, homology modeling, and crystallographic studies. Human NPC2 was expressed from baculovirus-infected *Trichoplusia ni* (Tn5) insect cells. The construct contained a hexahistidine purification epitope tag, and the protein was purified using Nickel affinity column chromatography. The purified protein was used in binding studies with dehydroergosterol (DHE), showing that human NPC2 was functional. Using the structure of bovine NPC2, we made a homology model and mapped the human mutations onto the model. Some modeled proteins, such as the V30M and S67P variants, are unclear as to how they lead to disease, thus a structure of the human protein would be

informative. Crystallization screens of human NPC2 were performed and led to crystals with a needle-like morphology, which diffracted to 4Å resolution. The structure of human NPC2 will be useful for understanding the mechanism of cholesterol binding and trafficking in cells, and to better understand the human metabolic disease NPC.

CONTENTS

	Page
ACKNOWLEDGMENTS	iv
ABSTRACT.....	v
LIST OF TABLES	viii
LIST OF FIGURES	ix
LIST OF ABBREVIATIONS	x
CHAPTER	
1. INTRODUCTION	1
2. RESULTS AND DISCUSSION.....	12
3. FUTURE DIRECTIONS	22
4. METHODS	24
APPENDIX: INSECT CELL CULTURE AND STERILE TECHNIQUE.....	27
BIBLIOGRAPHY.....	31

LIST OF TABLES

Table	Page
1. Mutations identified for NPC2.....	9
A.1. Contamination Results	29

LIST OF FIGURES

Figure	Page
1. Cholesterol traffic and Niemann-Pick disease	3
2. Bovine and human NPC2 sequence alignment	6
3. Molecular weight prediction of NPC2 gene	7
4. Bovine NPC2 structure and difference to human NPC2	8
5. Location of mutational effects on NPC2.....	9
6. Construct diagram.....	12
7. Insect cell culture and baculovirus expression.....	14
8. Western blot of expressed NPC2	15
9. Purification of NPC2. a) Purification Chromatogram	16
10. Fluorescent assay	18
11. Crystallized human NPC2.....	19
12. Diffraction images	20
13. Superimpose of sterol bound to NTD and NPC2 proteins.....	23
A.1. Fluorescence of DHE in aqueous and hydrophobic medium.....	30

LIST OF ABBREVIATIONS

LDL: low-density lipoprotein

NPC: Niemann-Pick type C disease

NPC1: gene and protein name of Niemann-Pick type C1

NPC2: gene and protein name of Niemann-Pick type C2; also used for particular disease name

NTD: N-terminal domain of NPC1 protein.

NPD: Niemann-Pick disease

α -NAGAL: α -N-Acetylgalactosaminidase

CHAPTER 1

INTRODUCTION

Cholesterol is a very well known molecule with a wide range of cellular functions. Cholesterol is essential for the synthesis of steroid hormones, vitamin D, bile acids and the synthesis of cellular membranes. Due to the key role that cholesterol plays in the health of cells a well-regulated and efficient means of transport is required for cholesterol homeostasis [1].

There are four mechanisms that maintain cholesterol homeostasis. First, cells can utilize enzymes to synthesize cholesterol. Secondly, cholesterol can be imported from outside the cell by endocytosis. Thirdly, cholesterol can be extracted from lipid droplets. Fourth, cholesterol can be removed from the cell membrane [2]. Two of the major sources of cholesterol in the human body are cholesterol absorbed from dietary intake in the intestine, and cholesterol synthesized in the liver. Even though every eukaryotic cell can synthesize cholesterol the majority of cell utilize cholesterol derived from other tissue, and it is important to see how cholesterol is delivered from one cell type to another cell type [3].

In the human body, cholesterol from the liver and the intestine form low-density lipoproteins (LDL), containing lipids, cholesterol, and protein. These lipoproteins are circulated through the circulatory system [3]. Cells containing the low-density lipoprotein (LDL) receptor recognize these lipoproteins [4]. After recognition of LDL by LDL receptors, clathrin complexes assemble to make clathrin-coated pits, which result in endocytosis [5]. The endocytosed vesicle is then fused to the early endosome [5]. In the early endosome, due to the acidic pH of the organelle, the LDL-receptor/lipoprotein

complex disassembles, and the LDL-receptor is transported back to the plasma membrane. Throughout the transport of the LDL-particle, the cholesterol being transported is in the esterified form (Figure 1). Esterified cholesterol has fatty acids attached to the cholesterol-3-oxygen. Once the LDL-receptor/lipoprotein complex disassembles, acid lipases hydrolyze the esterified cholesterol, replacing the fatty acid on the cholesterol-3-oxygen with hydrogen, resulting in unesterified cholesterol (Figure 1). Unesterified cholesterol then accumulates in the late endosome/lysosome [2]. As mentioned earlier, cholesterol is needed for synthesis of steroid hormones, vitamin D, bile acids and maintenance of cellular membranes, which require transport out of the late endosome/lysosome to the cytosol, endoplasmic reticulum, and mitochondria [2]. However, unesterified cholesterol cannot be transported by diffusion or active transport out of the endosome/lysosome, due to the fact that unesterified cholesterol is insoluble in an aqueous environment and is toxic to cells [1]. Hence, there must be a mechanism for removal of unesterified cholesterol from the late endosome/lysosome. Currently, the most clearly defined mechanism for cholesterol transport in the late endosome/lysosome involves the resident lysosomal proteins NPC1 and NPC2 (Figure 1). NPC1 and NPC2 are named after Niemann-Pick disease type C, a disease characterized by the accumulation of unesterified cholesterol in the late endosome/lysosome. NPC1 is a trans-membrane glycoprotein with 13 trans-membrane passes [6]. NPC1 has one luminal N-terminal domain, two large luminal loops, a smaller luminal loop, and several cytosolic loops [6]. NPC2 is soluble glycoprotein also known as HE1 [7]. It is known that NPC1 and NPC2 work in tandem to transfer unesterified cholesterol out of the late endosome/lysosome[8] (Figure 1). However, the mechanism of traffic outside of late

endosome/lysosome is not well understood.

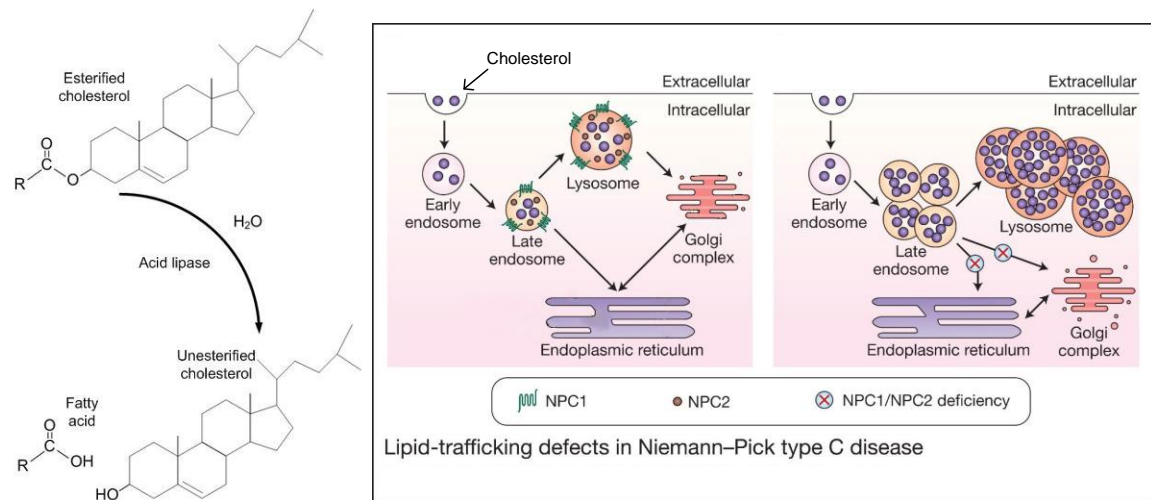


Figure 1: Cholesterol traffic and Niemann-Pick disease. Esterified cholesterols are delivered within low-density-lipoprotein (LDL) particles. When esterified cholesterols enter cell through LDL-receptor mediated endocytosis, vesicles go to early endosome, and esterified cholesterols are unesterified by acid lipase. Unesterified cholesterols accumulate in late endosome and lysosome. NPC1 and NPC2 work in tandem to transfer unesterified cholesterols out of late endosome/lysosome. If either of NPC1, NPC2 or both lose their function, unesterified cholesterols accumulate inside of late endosome/lysosome which seem to cause Niemann-Pick disease type C [9].

Mutations resulting in functionally defective NPC1 and/or NPC2 lead to the accumulation of unesterified cholesterol in the late endosomes/lysosomes (Figure 1). In NPC1 or NPC2 mutants, low cholesterol level in the ER membrane, with a threshold of 5% of overall ER lipids, leads to even more esterified cholesterols delivered to endosome [10]. When cholesterol levels are below the 5% of the threshold in the ER membrane, SREBP (Sterol regulatory element binding protein, a membrane protein) binds to SCAP (SREBP cleavage activating protein, also a membrane protein). Then the coat protein complex II (COPII) is recruited to make COPII-coated vesicles, which transport the complex to the Golgi complex. SREBP is then cleaved twice, and the liberated N-terminal fragment of SREBP is trafficked to the nucleus to trigger the transcription of

genes encoding the LDL-receptor and HMG-CoA reductase, one of the enzymes regulating cholesterol synthesis [1, 3, 10].

Accumulation of unesterified cholesterol results in Niemann-Pick type C disease (NPC). Symptoms and lifespans of individuals afflicted with this disease differ from patient to patient. With the exception of severe infantile onset patients, brain abnormalities are the most prominent symptoms followed by liver, spleen and lung abnormalities [9, 11-13]. NPC is an autosomal recessive lysosomal storage disease, a family of diseases characterized by the accumulation of substrates or ligands in the lysosome. Some of these diseases can be treated through enzyme/protein replacement therapy. This form of therapy involves the administration of the deficient enzyme/protein intravenously to a patient. The administered drug then traffics to the lysosome through the mannose-6-phosphate receptor or other pathway, where it reduces the dangerously high levels of the accumulated substrate/ligand. Interestingly, NPC patients have more problems than just accumulation of unesterified cholesterol. Liver and spleen samples of NPC patients often show accumulation of sphingomyelin, bis (monoacylglycerol) phosphate (LBPA), and glycolipids (essentially glucosylceramide, lactosylceramide, and ganglioside GM3). In the brain, alterations of glycosphingolipids (glucosylceramide, lactosylceramide, and gangliosides GM3 and GM2) become visible [12].

The structures of bovine NPC2 and its complex with a cholesterol analog have been determined [14, 15]. Because human NPC2 and bovine NPC2 have 80% sequence identity [14], a homology model of human NPC2 based on bovine NPC2 can have great predictive power. The bovine NPC2 has a seven beta-sheet sandwich structure, and its structure has revealed a pocket and two cavities that make a tunnel while it is bound to its

substrate [14, 15]. Likewise, our homology model predicts human NPC2 to share this seven beta-sheet sandwich structure. The bovine NPC2 complex with cholesterol-3-O-sulfate confirmed the fact that the pocket and the cavities widen to accommodate the substrate. Due to the hydrophobic ligand that NPC2 binds, the tunnel of bovine NPC2 is lined with hydrophobic residues, especially valine residues [15]. A comparison of the protein sequence of bovine and human NPC2 shows seven residues that are not conservatively substituted, four residues that are semi-conservatively substituted, and sixteen residues that are conservatively substituted (Figure 2). Non-conservatively substituted, semi-conservatively substituted and conservatively substituted residues were mapped on the bovine structure (Figure 4). The structure shows non-conservatively substituted and semi-conservatively substituted residues that are mostly on the outside of the protein, suggesting that the core of the protein is highly conserved.

	1									10									20							
Human	E	P	V	Q	F	K	D	C	G	S	V	D	G	V	I	K	E	V	N	V						
Bovine	E	P	V	K	F	K	D	C	G	S	W	V	G	V	I	K	E	V	N	V						
	21									30									★ 40							
Human	S	P	C	P	T	Q	P	C	Q	L	S	K	G	Q	S	Y	S	V	N	V						
Bovine	S	P	C	P	T	Q	P	C	K	L	H	R	G	Q	S	Y	S	V	N	V						
	41									50									60							
Human	T	F	T	S	N	I	Q	S	K	S	S	K	A	V	V	H	G	I	L	M						
Bovine	T	F	T	S	N	T	Q	S	Q	S	S	K	A	V	V	H	G	I	V	M						
	61									70									80							
Human	G	V	P	V	P	F	P	I	P	E	P	D	G	C	K	S	G	I	N	C						
Bovine	G	I	P	V	P	F	P	I	P	E	S	D	G	C	K	S	G	I	R	C						
	81									90									100							
Human	P	I	Q	K	D	K	T	Y	S	Y	L	N	K	L	P	V	K	S	E	Y						
Bovine	P	I	E	K	D	K	T	Y	N	Y	V	N	K	L	P	V	K	N	E	Y						
	101									110									★ 120							
Human	P	S	I	K	L	V	V	E	W	Q	L	Q	D	D	K	N	Q	S	L	F						
Bovine	P	S	I	K	V	V	V	E	W	E	L	T	D	D	K	N	Q	R	F	F						
	121									130																
Human	C	W	E	I	P	V	Q	I	V	S	H	L														
Bovine	C	W	Q	I	P	I	E	V	E	A																

Figure 2: Bovine and human NPC2 sequence alignment. Bovine and human NPC2 protein sequences are compared for their difference. The signal sequence is not shown. Different amino acid residues are colored. Orange color means not conserved substitution; yellow color means semi-conserved substitution; green means conserved substitution. Glycosylation state for each sequence are indicated by * sign. Human NPC2 has two glycosylations where as bovine NPC2 has one glycosylation.

There are three potential N-linked glycosylation sites in human and two in bovine NPC2.

A potential site at N19 (residue numbering starting from the mature human NPC2

sequence, not the beginning of signal sequence) in both human and bovine NPC2 do not

actually have carbohydrate added; it is speculated that the presence of a proline residue

following N19 prevents the addition of an N-linked glycan [16, 17] (Figure 2). Bovine

and human NPC2 have N-linked glycans at the N39 position (Figure 2), and these

glycans seem to be very important in delivering NPC2 to late endosome/lysosome; this

glycan is most likely the site at which the mannose-6-phosphate is attached resulting in trafficking to the lysosome [16-18]. Bovine NPC2 lacks the attachment of an N-linked glycan at N116, whereas human NPC2 does have an N-linked glycan attached at this position (Figure 2). It is not clear what function the N-linked glycan at residue N116 plays in human NPC2, but removal of this glycan does not affect its ability to transfer cholesterol [16, 17].

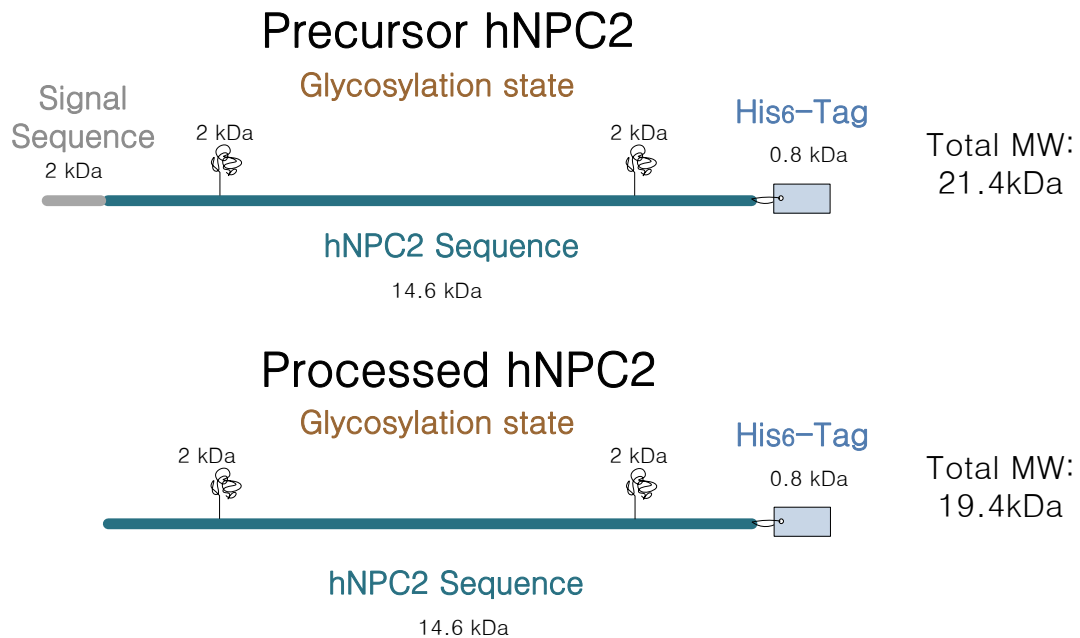


Figure 3: Molecular weight prediction of NPC2 gene. NPC2 protein sequence itself (Blue line) is 14.6kDa and the sequence has three possible glycosylation sites. However the first glycosylation site is not used [16, 17]. So, glycans are put to two glycosylation sites (Marked by curly cane). Also, the construct contain His₆ tag. Overall molecular weight is 21.4kDa for precursor human NPC2 or 19.4 kDa for processed human NPC2.

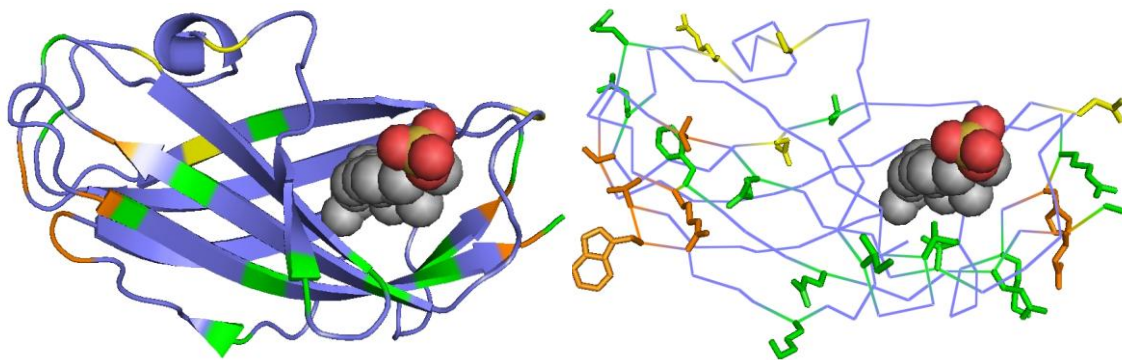


Figure 4: Bovine NPC2 structure and difference to human NPC2. Comparison between bovine and human NPC2, every difference in amino acid residues is colored on bovine structure. PDB ID 2HKA. Orange color means not conserved substitution; yellow color means semi-conserved substitution; green means conserved substitution. The molecule that is depicted with sphere is cholesterol-3-O-sulfate.

In spite of high similarity between bovine and human NPC2, human NPC2 structure is worth solving for two reasons. The first reason is to see the effects of disease-causing mutations on the structure. From NPC2 patients, a list of mutations is shown on Table 1. Many of the mutations result no expression or incomplete expression of NPC2 protein. Also, there are six missense mutations (Figure 5). Out of the mutations, there are two mutations, V30M and S67P that cannot be easily explained why they affect NPC2 function. Interestingly, residue 30 is not conserved between bovine and human NPC2. Yet, mutation to methionine produces disease in patients. By having the human wild type NPC2 structure and V30M and S67P mutant NPC2 structures, the effects from the mutation might be explained.

Genotype	Effect on protein		Reference
58G>T /58G>T	E20> X	E20> X	[7]
58G>T/331delA	E20> X	111 I N Y Q X	[7]
58G>T/27delG	E20> X	9 L S W R S A P L P R P W R C S S R T A V L W M E L X	[19]
352G>T/352G>T	E118>X	E118>X	[19]
199T>C/199T>C	S67>P	S67>P	[19]
IVS2+5G>A/IVS2+5G>A ^α	Various effects on protein		[19]
115G>A/115G>A	V39>M	V39>M	[20]
IVS1+2T>C/IVS1+2T>C ^α	Various effects on protein		[21]
58G>T/140G>T	E20> X	C47>F	[21]
278G>T/278G>T	C93>F	C93>F	[21]
90G>A/ Undefined ^β	V30>M	Undefined	[21]
172C>T/172C>T	Q45>X	Q45>X	[22]
141C>A/141C>A	C47>X	C47>X	[22]
295T>C/295T>C	C99>R	C99>R	[22]
338C>T/338C>T	P120>S	P120>S	[23]
436C>T/436C>T	Q146>X	Q146>X	[23]
165C> A or G/165C> A or G ^γ	Y55>X	Y55>X	[23]

Table 1: Mutations identified for NPC2. The table lists genotypical and phenotypical changes from mutations. DNA numbering in genotype starts from first nucleotide residue of cDNA that does not include intron sequence. Protein numbering in phenotype starts from first amino acid residue of NPC2 containing signal sequence. ^α) Splice site mutation results various NPC2 mutant sequences. ^β) Heterozygous mutation that one of alleles was found and yet the other is not found to be defined. ^γ) The literature does not define mutation specifically.

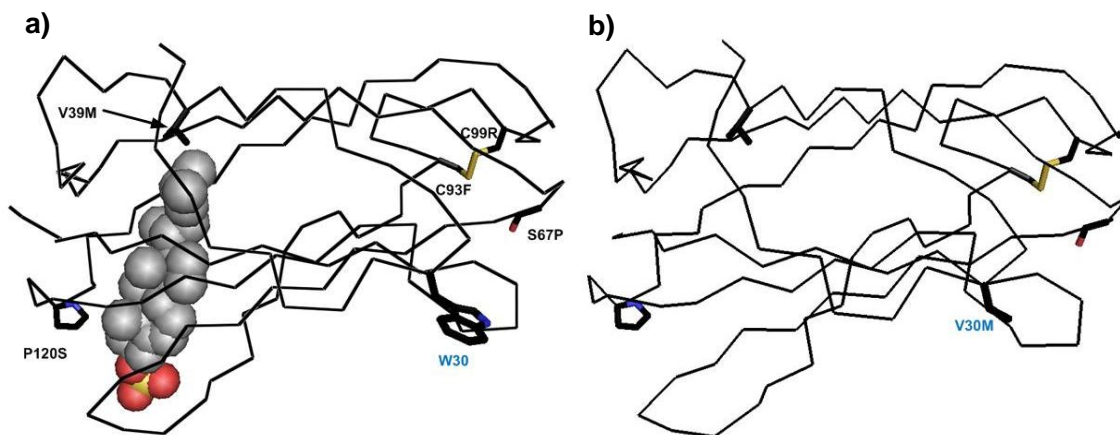


Figure 5: Location of mutational effects on NPC2. From the table 1, substituting mutations are highlighted by showing side chains and by labeling protein code and residue number. The protein code before residue number is wild type amino acid residue, and the protein code after residue number is substituting amino acid due to mutation. A) Substituting mutation effected residues are highlighted on bovine NPC2 structure. PIB ID 2HKA. “W30” is highlighted blue because the residue is different between bovine and human, yet mutation of the residue in human cause NPC2 disease. B) Substituting mutation effected residues are highlighted on homology model of human NPC2. Based structural information from PIB ID 2HKA. On the human NPC2 model, “V30M” is highlighted blue for its residue number is same as W30 in bovine NPC2.

A second reason is to understand how NPC2 bind and transfer cholesterol. As mentioned earlier, the trafficking of unesterified cholesterol outside of the late endosome/lysosome is not well understood. By understanding how NPC2 binds and transfers cholesterol, trafficking of cholesterol can be better understood. Because many of molecular and cellular experiments are done with human NPC2, the human NPC2 protein is a good candidate for structural studies.

In order to express a recombinant protein, there are several organisms that may be used: bacteria, yeast, insect cells, and mammalian cells. Each of these systems has their advantages and disadvantages. For the expression of human NPC2 protein, we have chosen the baculovirus expression system in insect cells for several reasons. Insect cells are often used to express proteins that require post-translation modifications. In previous

studies we have used various expression systems for other lysosomal proteins such as α -N-Acetylgalactosaminidase (α -NAGAL). Attempts were made to express NAGAL in an *Escherichia coli* system as well as a *Kluyveromyces lactis* yeast system. We saw solubility problems in *E. coli* expressed NAGAL and saw hyperglycosylation in *K. lactis* expressed NAGAL [24]. However, when NAGAL was expressed using an insect cell expression system, using *Trichoplusia ni* (Tn5) insect cells, these problems did not occur. Also, from the α -NAGAL structure resulting from crystallization of protein purified from insect cells, an additional advantage was observed. Additional crystal contacts were observed resulting from the pauci-mannose carbohydrates used by Tn5 insect cells [24]. Due to the success we experienced using insect cells to express α -NAGAL, we reasoned that insect cells were the ideal expression system for NPC2.

In order to solve the structure of human NPC2, we have chosen X-ray crystallography. NPC2 protein was produced through a baculovirus express system. A His6 tag on C-terminus of NPC2 was useful tool for purification. Then the protein was tested for its proper folding and proper binding capability in a fluorescence-based binding assay. After binding assay, NPC2 were screened in crystallization experiments. Crystals from the screen were shot at a synchrotron X-ray source and showed diffraction to 4Å resolution.

CHAPTER 2

RESULTS AND DISCUSSION

In order to express recombinant NPC2 in insect cells we decided to use a baculovirus expression system. We opted to use the Bac-n-blue system from Invitrogen. In this system a gene of interest has to be incorporated into a donor plasmid, pFastbac-DUAL (Figure 6). This can be done through LR recombination, which switches a *ccdB/chloramphenicol* cassette from the pFastbac-DUAL-DEST vector with the *NPC2/Ampicillin* cassette in an entry vector, pCR8. The resulting plasmid has *NPC2* under the regulation of the polyhedrin promoter flanked by two Tn7 recombination sites. The donor plasmid was then used to transform DH10Bac™ *E. coli* cells (Figure 6). The DH10Bac™ cells contain an inactivated baculovirus genome as well as a helper plasmid containing the Tn7 recombinase gene used to recombine portion of the donor plasmid with the baculovirus genome, resulting in an activated baculovirus containing *NPC2* under the regulation of the polyhedrin promoter.

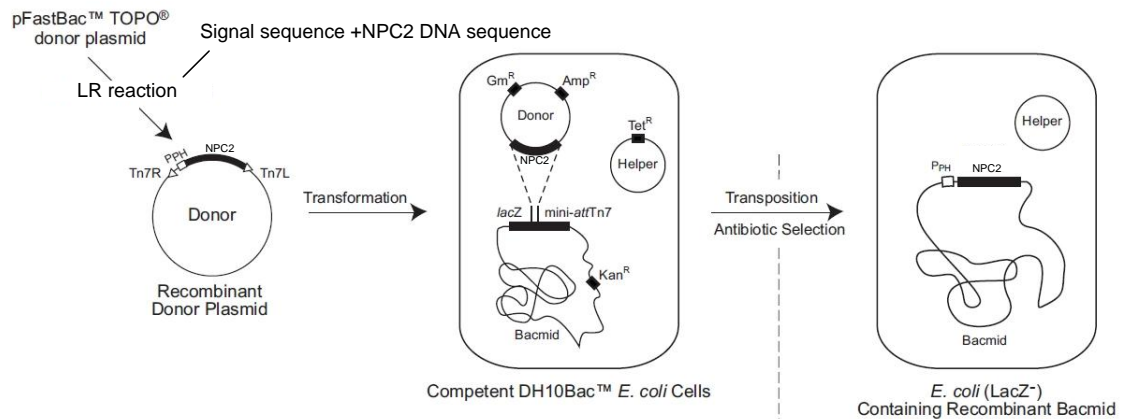


Figure 6: Construct diagram. Legend on next page

Figure 6: Construct diagram. human NPC2 that has natural signal sequence and has six histidines as a tag is placed in pFastbac[™] Dual plasmid, a donor plasmid, by LR reaction kit from Invitrogen. The plasmid has polyhedrin promoter (P_{Ph}). The recombinant NPC2 gene was placed after polyhedrin promoter. Upon transformation of pFastbac[™] Dual plasmid containing recombinant NPC2 gene to DH10Bac[™], polyhedrin promoter and recombinant NPC2 gene are transferred to bacmid plasmid. The bacmid plasmid is used for co-transfection of insect cells. The figure is modified from a figure in Invitrogen manual (Catalog number A10606).

Once the recombinant baculovirus was generated, transfection of *Spodoptera frugiperda* (Sf9) insect cells was performed. The initial virus stock generated from this transfection was of insufficient titer, concentration of virus, and volume for large-scale expression. In order to generate a high titer and high volume baculovirus stock, a number of rounds of virus amplification were performed in Sf9 cells (Figure 7). Each round of amplification was performed using an approximate multiplicity of infection (MOI) of .1 plaque forming units / cell. A total of four rounds of amplification were performed with an increase of volume of 5mL to 500mL occurring in the fourth round of amplification. The titer of each round of amplification was estimated using cell diameter, and secreted levels of NPC2 after infection of Tn5 cells as a metric. The Tn5 cell line, BTITn-5B1-4, derived from *Trichoplusia ni* embryonic tissue was used for expression due to its ability to express and secrete proteins at a higher level than SF9 cells. Secreted levels of NPC2 were measured using anti-6x-His western blots. Once a high titer and high volume virus stock was created, Tn5 cells were infected in order to express secreted NPC2. Infections were carried out with an approximate MOI of 3, and the infection was allowed to progress for three days before harvesting the supernatant.

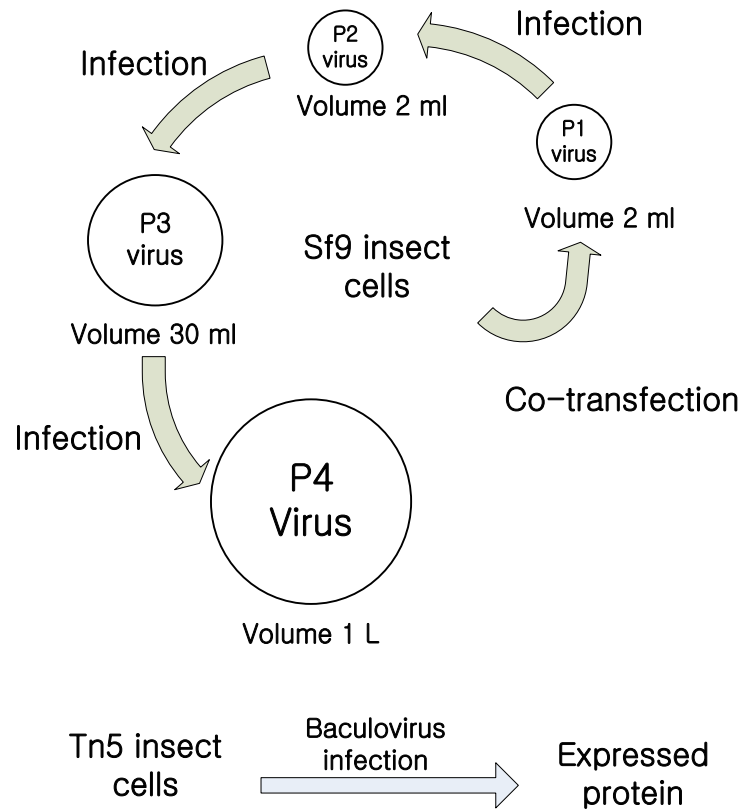


Figure 7: Insect cell culture and baculovirus expression. Before expression of protein by viral infection, healthy wild type insect cells, Sf9 and Tn5, were maintained. With maintained Sf9 cells, baculovirus is expressed and assembled inside of cells and lyse out of cells. Volume of virus from co-transfection is about 2ml, and titer ranges from 1×10^6 to 1×10^7 pfu (plaque-forming-unit)/ml. However, for large scale infection, you need more volume and higher titer. Amplification of the virus using Sf9 cells bring volume and titer up during infection. With enough viruses, wild type Tn5 cells are used to express protein.

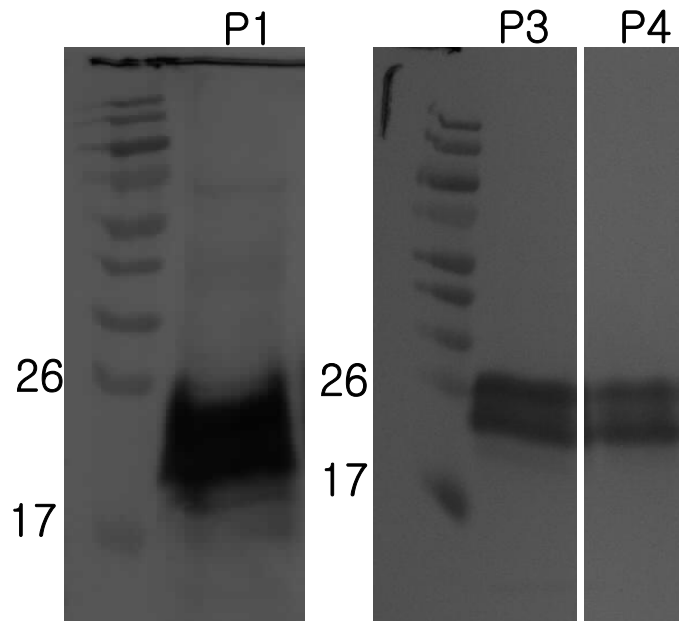


Figure 8: Western blot of expressed NPC2. Virus generation zero, P1, is virus that was produced from co-transfection of Sf9 cells. P3 and P4 is virus that was amplified by infecting Sf9 cells for second times and third times. To test viral presence, each virus generation were used to infect Tn5 cells to express NPC2. And supernatant from the infections are used to run Anti-his western blot. Coomassie staining SDS-Gel was not done at this stage because the level of protein expression was too low.

Large-scale infection of Tn5 cells was difficult due to contamination of the cultures. Overall, almost 6 liters of infection supernatant were harvested. The harvested infection supernatant was buffer exchanged with nickel column binding buffer (250mM NaCl, 50mM phosphate pH 7.0, .01% azide), and concentrated to 400ml. The purification was done using a 5 ml column of Nickel charged resin (Ni-NTA). NPC2 was eluted from the column using a linear elution profile with nickel column elution buffer (250mM NaCl, 50mM phosphate pH 7.0, .01% azide, 400mM imidazole). NPC2 eluted after most of the proteins in the supernatant (Figure 9a). Fractionation of the elution was done with a fraction volume equal 5 ml. Exact fraction numbers where NPC2 eluted on that purification were from 37 to 46 (Figure 9b). Those fractions were combined, buffer

exchanged to 20mM ammonium acetate, pH4.5, and concentrated.

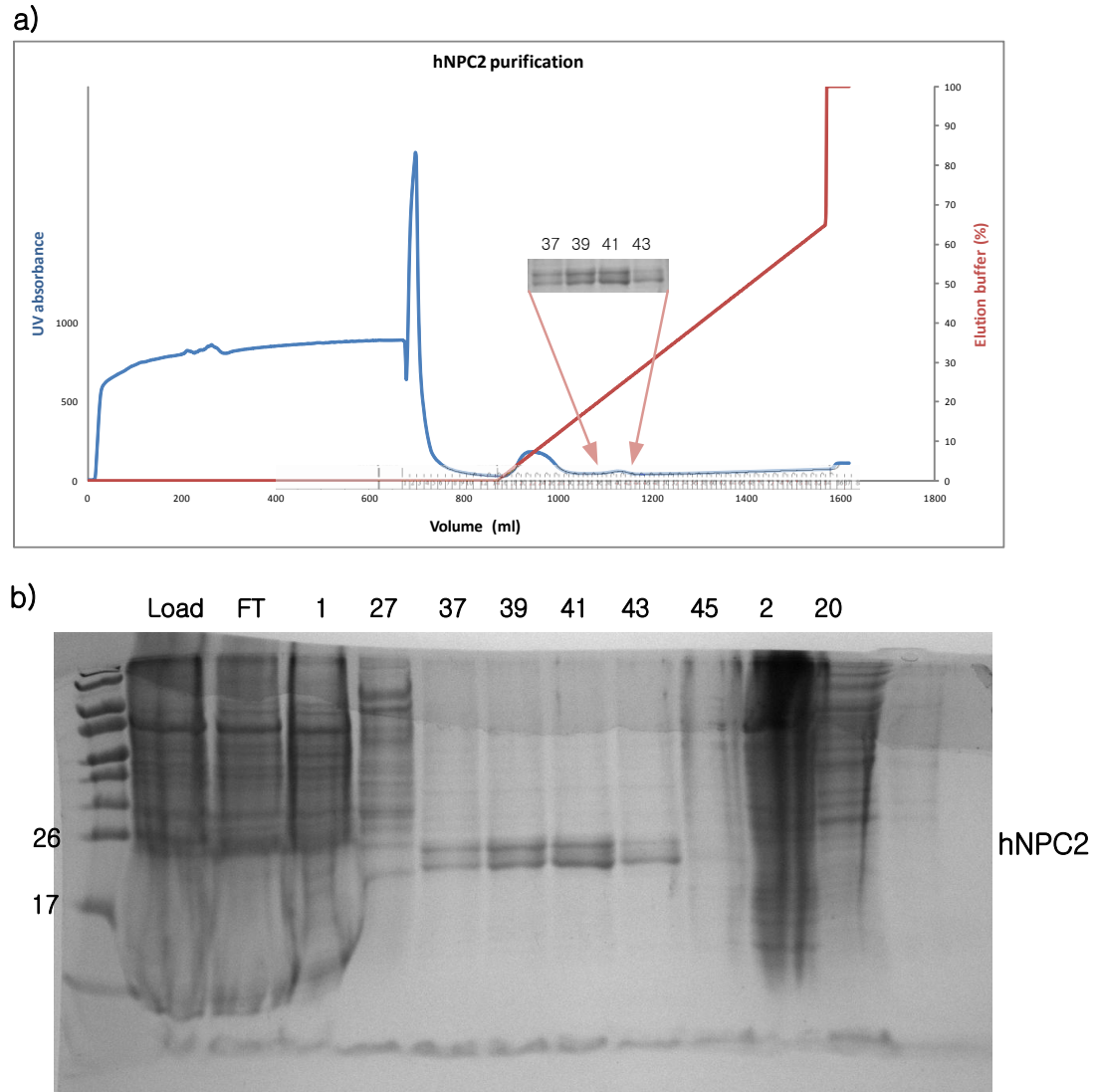


Figure 9: Purification of NPC2. a) Purification Chromatogram. Using FPLC with 5ml Ni^{2+} -NTA column, NPC2 was purified. Wash buffer was 250mM NaCl and 50mM phosphate buffer, with an elution buffer has 400mM imidazole, 250mM NaCl, and 50mM phosphate buffer. The gradient was from 100% wash buffer to 40% wash buffer and 60% elution buffer. b) Fraction samples loaded Coomassie stained gel. Fractions from FPLC purification were used to run SDS gel stained with coomassie-blue. Each fraction was 5ml and fraction from 37 to 46 was combined after the gel.

The purified NPC2 was then subjected to a cholesterol-binding assay. The purpose of the binding study was to test the insect cell expressed human NPC2 for its ability to bind cholesterol in order to prove that the protein was active. If the protein is correctly folded then it should be able to bind fluorescent cholesterol, and show a change in the fluorescence spectrum [14]. The binding assay was performed by incubating dehydroergosterol (DHE) with NPC2, in binding buffer (20 mM citrate pH 5.5, 150 mM NaCl). Upon binding of DHE to a cholesterol binding protein, such as NPC2, the DHE fluoresces after excitation due to a conjugated triene system; however, the fluorescence is quenched by an aqueous environment [25, 26]. Figure 14 (in the appendix) might seem to contradict the previous statement, but there have been observations of dimerization of DHE and of low quantum yield in chloroform [27, 28]. Comparing the spectrum from Friedland, Natalia et al. [14] to our data (figure 10c) a similar trend in fluorescence is observed. Also, the negative control of our NPC2 alone and DHE alone in binding buffer shows minimal fluorescence. The reason why NPC2 alone has an increase in fluorescence around 345nm is due to light scattering of NPC2 or other small particles. The fluorescence data shows that human NPC2 expressed by insect cells is able to bind unesterified cholesterol, and is therefore active.

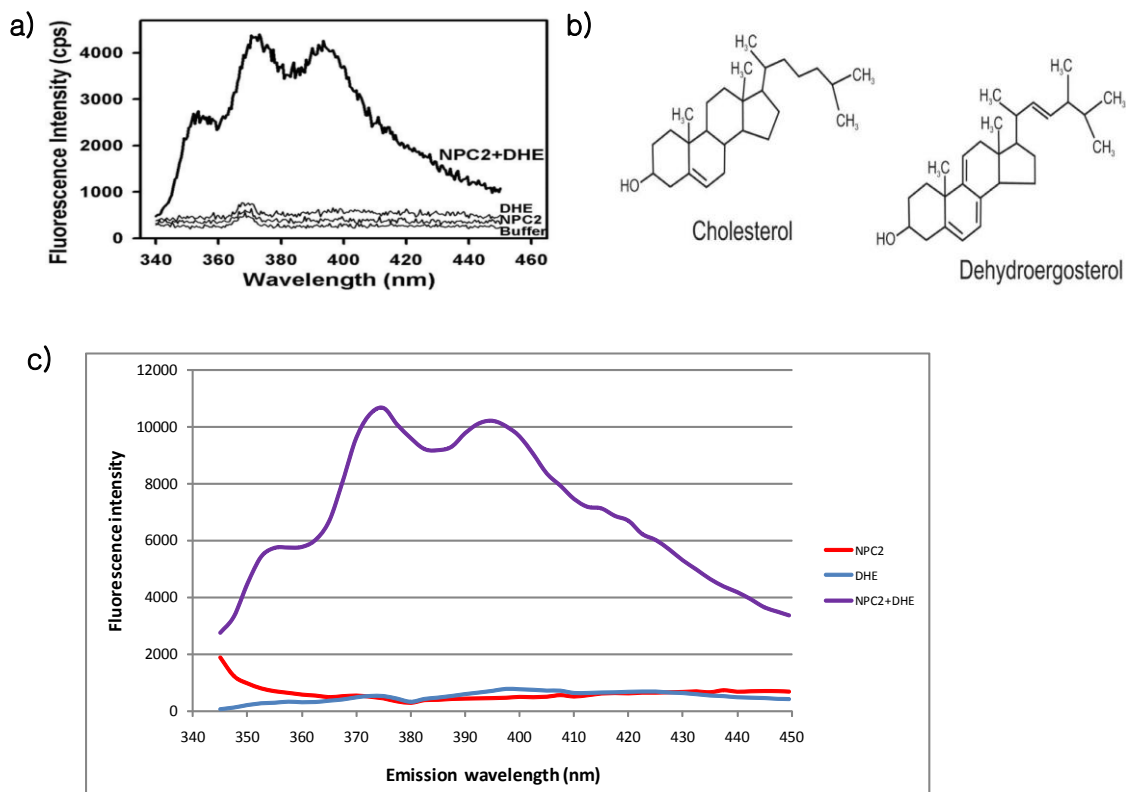


Figure 10: Fluorescent assay. a) NPC2 and DHE complex fluorescent spectra of Natalia et al. [14]. Buffer (20 mM citrate/150 mM NaCl, pH 5.0) was mixed with NPC2 alone (final concentration 10 μ M), DHE alone (final concentration 1 μ M), and human NPC2/ DHE (final concentration NPC2: 10 μ M and DHE: 1 μ M). b) chemical structure of cholesterol and dehydroergosterol (DHE) [29]. c) NPC2 and DHE complex fluorescent spectra. Buffer (20 mM citrate/150 mM NaCl, pH 5.5) was mixed with human NPC2 alone (final concentration 10 μ M), DHE alone (final concentration 1 μ M), and human NPC2/ DHE (final concentration NPC2: 10 μ M and DHE: 1 μ M).

Once we confirmed that our purified NPC2 was biologically active we initiated crystallization screening. When human NPC2 was screened with the Precipitant Synergy screen (Emerald BioSystems), most of the crystallization conditions showed a sign of precipitation shortly after the drop was set-up. However, one condition (46) showed small crystals after three weeks at room temperature (Figure 11a, b). In a follow-up experiment, the condition was diluted to 60% strength, and improved crystals were observed (Figure 11c). This result indicates that by lowering the concentration of one or more of the

components the rate of nucleation is slowed. By lowering the nucleation rate, fewer nucleation events occur, which results in larger crystals.

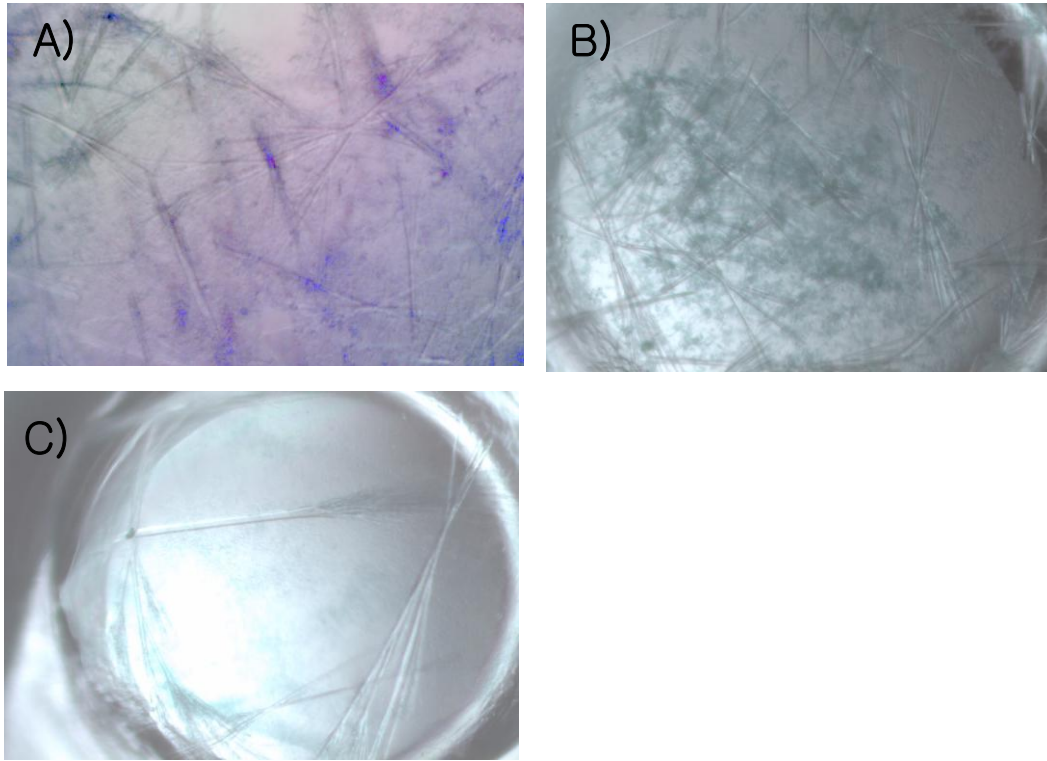


Figure 11: Crystallized human NPC2. NPC2 were crystallized in condition a-c) 16.75% (w/v) PEG 3350, 10.05% (v/v) isopropanol, 0.2M $(\text{NH}_4)_3$ Citrate/Citric acid pH 4.5. a) was crystallized during screen and it was confirmed by crystallizing b) was in same condition again. Was crystallized with same condition but diluted that condition with 20mM ammonium acetate pH4.5 (precipitant: ammonium acetate = 6:4).

We then set out to determine if the crystals obtained through screening were in fact protein crystals and not inorganic salt crystals. In order to determine if the crystals we grew were salt or protein, we decided to look at the diffraction pattern of the crystal. The diffraction data lets us determine whether the crystal is protein or salt. Salt only diffracts to high resolution, but if the crystal contains macromolecules, such as protein,

then low-resolution spots should be present. Depending on the crystal quality, high-resolution diffraction patterns may or may not be visible for protein crystals. So, in order to see whether the crystal is protein or salt, low-resolution diffraction patterns in diffraction image will show the difference.

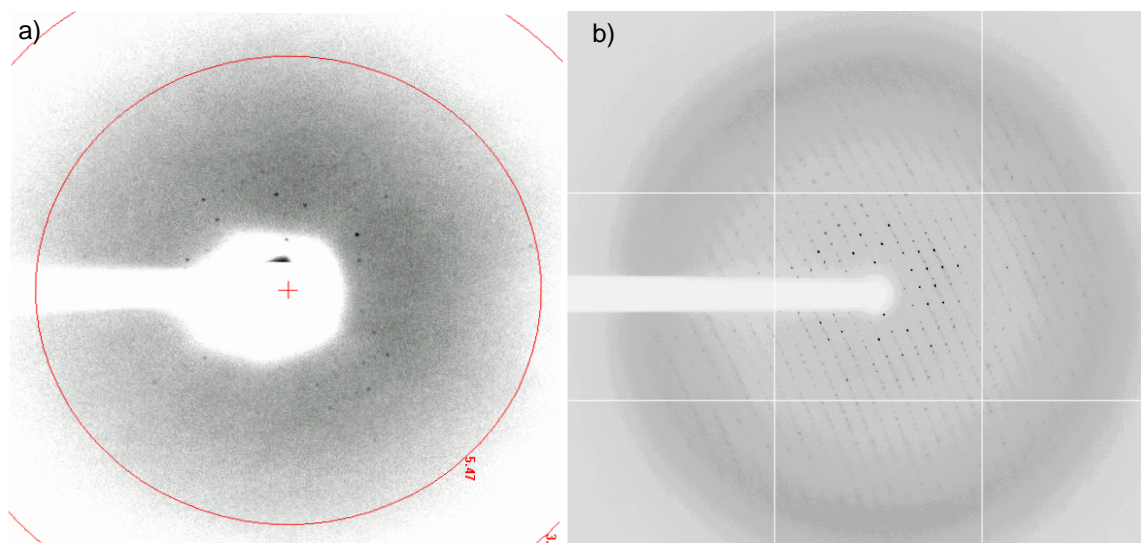


Figure 12: Diffraction images. a) A diffraction image that was from a crystal, shown in figure 11a. The X-ray beam was from RU-H3R generator (Rigaku) b) A diffraction image that was from a crystal shown in Figure 11c. The X-ray beam was from micro-beam of Argonne Advanced photon source in Chicago, IL.

The initial crystals from the Precipitant Synergy screen (Emerald BioSystems) condition 46 (figure 11a) were harvested and shot with the in house X-ray generator. An image of diffraction data is shown on figure 12a. Even though there are mostly faint spots, a few brighter spots are observed at low resolution. This data allowed us to confirm that the crystals are in fact protein and diffracted to approximately 8\AA . The optimized crystals from the diluted condition 46 (Figure 11c) were exposed to shot at a much brighter X-ray source, the Argonne National Laboratory synchrotron. The brighter synchrotron x-rays

showed that the crystals diffracted to 4Å resolution (Figure 12 b).

CHAPTER 3

FUTURE DIRECTIONS

The human NPC2 crystal should be optimized to make bigger crystals in order to improve the diffraction images. Also, the V30M and S67P mutants (Figure 5) can be expressed and crystallized. Comparison of the wild type human NPC2 to the mutant NPC2 structures might reveal the molecular basis for disease in patients with those mutations. In order to understand how NPC2 interacts with NPC1, NPC2 can be co-crystallized with N-terminal domain (NTD) of NPC1. The human NPC1 NTD (which binds cholesterol) has been expressed in baculovirus-infected insect cells and crystallized [30], so that both proteins are available for co-crystallization experiments. Moreover, co-crystallization experiments with and without cholesterol can reveal the mechanism of binding and transfer of cholesterol between NPC1 and NPC2. The co-crystal structure may show that the cholesterol binding pockets of each protein do not interact; even so, this would be an interesting result. Mutagenesis of NPC1 NTD has identified the regions on that protein responsible for the interaction with NPC2, so it may be possible to mutate these residues to make a tighter interaction more suitable for crystallization experiments.

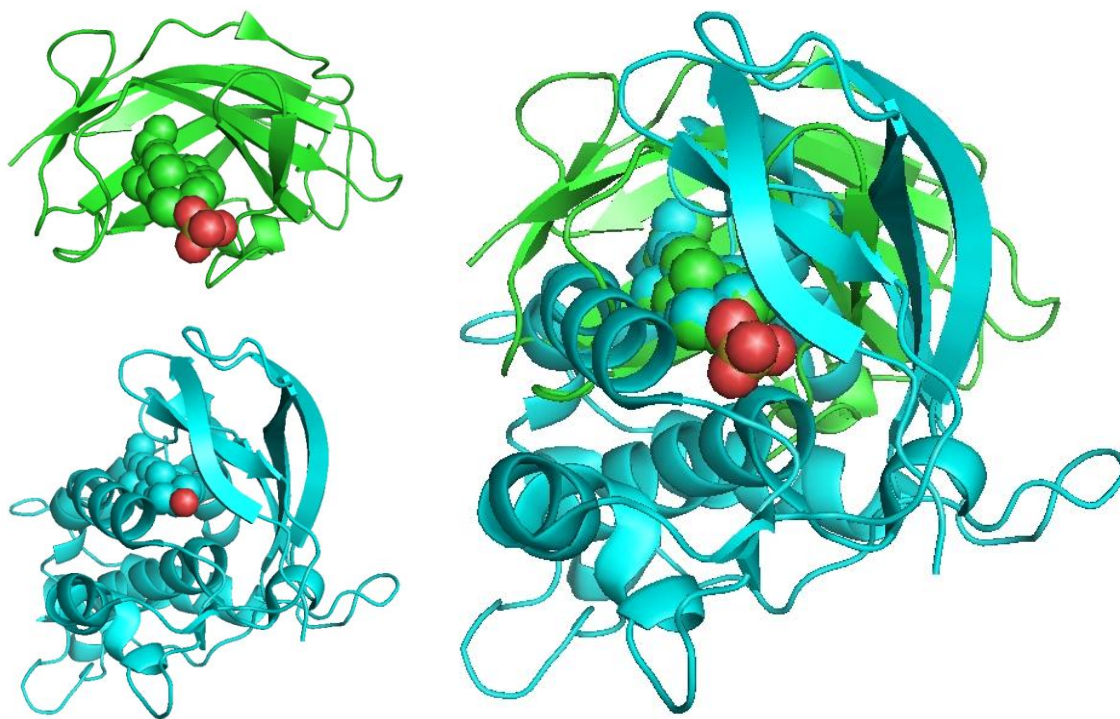


Figure 13: Superimpose of sterol bound to NTD and NPC2 proteins. The cyan molecules are NTD of NPC1 protein structure complex with cholesterol (PDB ID: 3GKI). The green molecules are NPC2 protein structure complex with Cholesterol-3-O-sulfate (PDB ID: 2HKA). Those molecules are superimposed upon sterols that each of them bound.

CHAPTER 4

METHODS

Plasmid construct/ NPC2 expression

The NPC2 gene (including the native signal sequence) was inserted by Nilima Kolli into a bacmid via a LR reaction and transformed into *Escherichia coli* cells. The construct included a C-terminal His6. The LR reaction uses site specific recombination by utilizing bacteriophage lambda process. The gene, flanked by attL1 and attL2 sequences, cross with ccdB, flanked by attR1 and attR2 sequences. Removal of the suppressor ccdB gene allows the *E. coli* to grow. In Gateway LR clonase II mix from Invitrogen, integrase and excisionase enzymes conduct the reaction. The recombinant bacmid was used to co-transfect Sf9 cells to produce virus that has NPC2 gene. Sf9 cell line is derived from ovarian tissue of the fall armyworm, *Spodoptera frugiperda*. First amplification of virus was done through adding 500 μ l of first virus generation (P1) to 2ml of 9×10^5 Sf9 cells/ml. Later amplifications were done by adding 16.7ml virus/1L insect culture. For protein expression, Tn5 cells were infected with the viruses that were produced by Sf9. Tn5 cell line, BTITn-5B1-4, is derived from originally the *Trichoplusia ni* embryonic tissue. Volume of virus used was with same ratio as amplification of virus, 16.7ml virus per 1L insect culture. After an infection, expression level was confirmed by western blot using either anti-NPC2 or anti-His6 as primary antibodies.

Purification

Prior to purification, the infection supernatant containing NPC2 was concentrated and buffer exchanged nickel binding buffer (250 mM NaCl, 50mM sodium phosphate pH 7, and .01% azide). The His6-tag on the C-terminus of NPC2 was used for affinity purification of the protein using nickel-nitrilo-triacetic acid resin (Ni²⁺-NTA) column

chromatography. Wash buffer was 250 mM Sodium chloride and 50mM phosphate buffer pH 7. 100% elution buffer is 400mM imidazole, 250mM NaCl, and 50mM phosphate buffer. A 5ml column was utilized. Elution fractions were collected in 5mL increments. After purification, fractions 37 to fraction 46 were combined, and buffer was exchanged with 20mM ammonium acetate, pH4.5. The buffer exchanged protein solution was concentrated to roughly 500 μ l final volume. The final concentration of NPC2 was determined to be 16.8mg/ml by Abs280 measurements.

NPC2 protein binding assay

The protocol for the binding assay was adapted from Friedland, Natalia et al [14]. Intensity of fluorescence was measured using a Fluorolog 3-21 spectrometer equipped with a 450W xenon arc lamp, a double excitation monochromator, a single emission monochromator, and a cooled PMT [31]. The buffer was 20 mM citrate and 150 mM NaCl, pH 5.5. DHE was dissolved in pure methanol (optima) and the concentration was measured at 324nm. The final concentration of NPC2 in the reaction was 10 μ M, and DHE concentration was 1 μ M. As a negative control, buffer alone and NPC2 protein alone with buffer and DHE alone with buffer were prepared. The samples were incubated for at least an hour at 4°C prior to the fluorescence measurement. The samples were then transported to a quartz microcell. Then the fluorescence was measured using an excitation wavelength 338 nm with band pass slit of 0.5 nm. Fluorescence emission was measured from 340nm to 450nm with band pass slit of 0.5 nm.

Crystallization/ Optimization of NPC2 crystal

Using the Precipitant Synergy crystallization kit (Emerald BioSystems), 1 μ L of purified human NPC2, at a concentration of 8 mg / mL, was mixed in a ratio of 1:1 with the crystallization buffers contained in the kit, and then added to a sitting drop

crystallization tray. The initial crystals formed in condition 46 (16.75% (w/v) PEG 3350, 10.05% (v/v) isopropanol, 0.2M (NH₄)₃ Citrate/Citric acid pH 4.5). To optimize the crystals the initial condition was diluted with 20mM ammonium acetate pH4.5 to 60% of the original concentration.

APPENDIX

INSECT CELL CULTURE AND STERILE TECHNIQUE

In baculovirus expression, large scale expression depends on scaling wild type insect cells up from small volumes. Usually small cultures such as adherent cultures of 5ml or 15ml or suspension cultures of 30ml are the start up volume. Depending on temperature of culture room or depending on which shakers you are using, the cell growth rate can be different, but usually cells go to confluence about three to four days. So, large scale culture of 1L means approximately 2 weeks of preparation before. If you get any contamination in the 1L culture, you might have to go back to small scale in worst scenario. For the reason, set ups are usually done in duplicate and small scale cultures are always maintained just in case. Back up cultures are there to save time if contamination happens to affect actual experiment cultures. However, without sterile technique, back up cultures are also most likely to be contaminated. The techniques are as following: Before starting any culture, sterilize each flask right before they will be used. This reduces the likelihood that a possible contaminant will diffuse into the flask. Before sterilization, the flask should be examined for damage to the flask or the cap filters. If the flask itself is damaged, the flask is thrown away; if the cap's filter is damaged, the top of cap is covered with autoclave tape to plug any holes. After sterilization and while the flask are cooling, culture hood's UV is turned on for 15 minutes and hood is turned to equilibrate air inside for 5 to 10 minutes to prevent any contaminant inside the hood going into the cultures. When that preparation is all done, experiment actually starts. With 70% ethanol, wipe work area and put ethanol on hands and arms. During experiments,

hands are avoided above the opened flask, and flasks are tilted to avoid too much air blowing down culture, especially for flasks that have large brim. Careful technique still does not avoid contamination entirely; there are other factors involved.

Interpretation of contamination data

Because of prolonged contamination, detailed data were collected in order to detect patterns of contamination. The detailed data started from April 17th 2010 and ended September 3rd 2010. During the time, there had been 218 experiments of different cell type and volume. Overall, we saw 10 occurrences of contamination, leading to overall contamination rate of about 4.6%. Even though the rate is very low, the rate is still higher than expected. If volumes of 30mL and less are excluded, the rate increases to 10.5%. Moreover when volume of 30mls or less are excluded, the contamination rate of Tn5 cells increases a little bit to 10.6%. All of the contamination was from bacteria. So, assuming that bacterial growth rate is fast, expectation is 24 hours are more than enough time for them to take over. And when bacteria take over, culture gets cloudy and smells distinctively different. Among ten contaminations only two or possibly three were able to take over the culture within a day without my intervention. Others took more than two days to take over the culture without my opening the flask. That could mean many things but if I have to pick reason why that might have happened, I can tell two things. A first possibility is contaminant might have been introduced somehow while they were shaking. A second possibility is those bacteria might take longer than typical to grow.

Date	Cell type	Volume (ml)	Set up	Contamination	Take over time (Day)
April [4]					
20	Sf9	30	8	1	1
22	Sf9	200	6	1	4
26	Sf9	200	5	1	2
May [5]					
5	Tn5	450	2	1	2
26	Tn5	30	8	1	2
July [7]					
20	Tn5	600	1	1	3
August [8]					
2	Tn5	600	2	1	2
5	Tn5	600	3	1	1
29	Tn5	600	5	2	3 and 4

Table A.1: Contamination results. From April 17th 2010 to September 3rd 2010, 218 set ups regardless of cell type and volume of set up are done. Over all contamination that occurred during that time is 10 occurrences. Cell type is cells that were used to set up. Volume of cultures is volume of media plus cells for the set up. Contamination section indicate number of contaminated flask out of set ups done on the date. Take over time is rough estimate of time contaminant took to take over whole flask.

Dehydroergosterol

Dehydroergosterol (DHE) is a small molecule that is analog of cholesterol. As mentioned previously, DHE fluoresces when it is surrounded in a hydrophobic environment. In order to make sure of the fact, DHE had been added to pure methanol, pure chloroform and 20 mM citrate/ 150 mM NaCl buffer. Figure 14 shows that pure methanol have higher fluorescence than the buffer which is expected for the buffer is aqueous environment. But in pure chloroform, DHE was expected to fluoresce for chloroform is hydrophobic. However, literature reports show the dimerization of DHE and of low quantum yield in chloroform [27, 28] suggest the quenching affect from dimerization. So, considering only DHE in pure methanol, DHE does fluoresce in

hydrophobic environment. With the result, if DHE fluoresce when DHE is combined with NPC2, which means DHE is bound to NPC2 for the binding channel is hydrophobic.

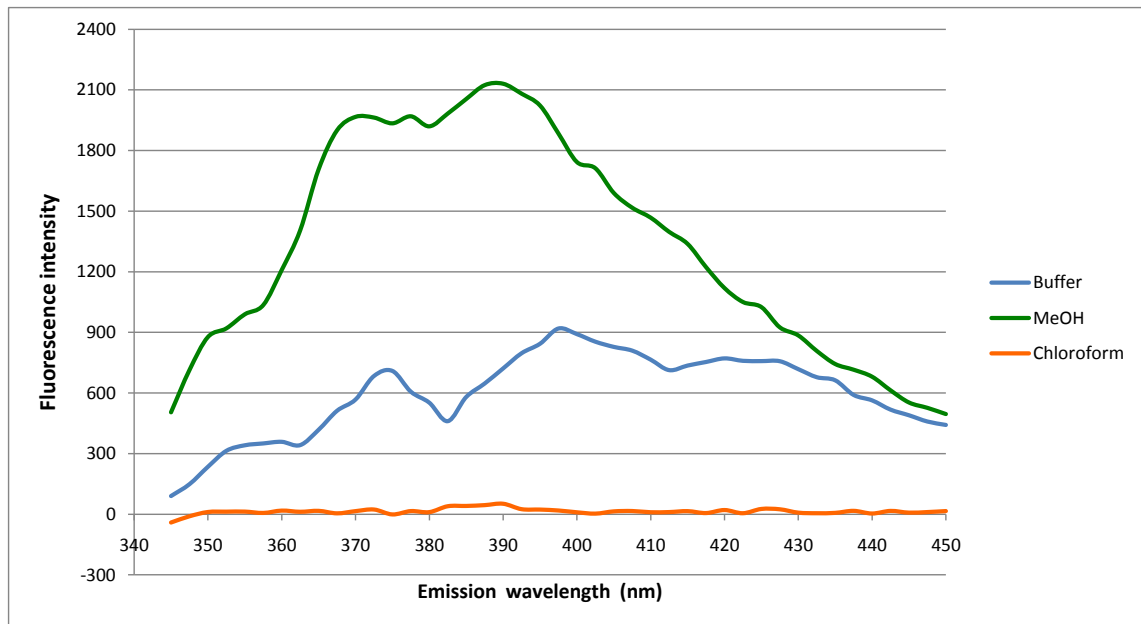


Figure A.1: Fluorescence of DHE in aqueous and hydrophobic medium. Fluorescence spectra of DHE in various medium. Buffer (20 mM citrate/150 mM NaCl, pH 5.5), optima pure methanol and optima pure chloroform were mixed with DHE alone which make DHE concentration 1 μ M concentration.

BIBLIOGRAPHY

- [1] Liu, Jun-Ping. New function of cholesterol binding proteins. *Molecular and Cellular Endocrinology*. 2009; 303 : 1-6.
- [2] Hu, jie et al. Cellular cholesterol delivery, intracellular processing and utilization for biosynthesis of steroid hormones. *Nutrition and metabolism*. 2010; 47.
- [3] Cox, Michael M. and David L. Nelson. *Lehninger Principles of Biochemistry*. 4th ed. New York: W.H, Freeman and company, 2005.
- [4] Brown MS and Goldstein JL. A receptor-mediated pathway for cholesterol homeostasis. *Science*. 1986; 232: 34-47.
- [5] Alberts, Bruce et al. *Molecular biology of the cell*. 5th ed. New York: Garland science Taylor and Francis group, 2008.
- [6] Ioannou, Yiannis A. The structure and function of the Niemann-Pick C1 protein. *Molecular genetics and metabolism*. 2000; 71: 175-181.
- [7] Naureckiene, Saule et al. Identification of HE1 as the second gene of Niemann-Pick C disease. *Science*. 2000; 290: 2298-2301.
- [8] Infante RE et al. NPC2 facilitates bidirectional transfer of cholesterol between NPC1 and lipid bilayers, a step in cholesterol egress from lysosome. *Proc. Natl. Acad. Sci. U.S.A.* 2008; 105: 15287-15292.
- [9] Pacheco, Chris D. and Andrew P. Lieberman. The pathogenesis of Niemann-Pick type C disease: a role for autophagy? *Expert review in molecular medicine*. 2008; 10 (e26).
- [10] Burns MP and Rebeck GW. Intracellular cholesterol homeostasis and amyloid precursor protein processing. *Biochemica et Biophysica Acta*. 2010; 1801: 853-859.
- [11] Sévin M et al. The adult form of Niemann-Pick disease type C. *Brain*. 2007; 130: 120-133.
- [12] Vanier MT. and Millat G. Niemann-Pick disease type C. *Clin Genet*. 2003; 64: 269–281.
- [13] Vanier MT. Niemann-Pick disease Type C. *Orphanet Journal of Rare Diseases* 2010; 5 (16).

- [14] Friedland, Natalia et al. Structure of a cholesterol-binding protein deficient in Niemann-Pick type C2 disease. *PNAS*. 2003; 100 (5): 2512-2517.
- [15] Xu, Sujuan et al. Structural Basis of Sterol Binding by NPC2, a Lysosomal Protein Deficient in Niemann-Pick Type C2 Disease. *Journal of Biochem*. 2007; 282 (32): 23525-23531.
- [16] Chikh, K et al. Niemann-Pick type C disease: importance of N-glycosylation sites for function and cellular location of the NPC2 protein. *Molecular Genetics and Metabolism*. 2004; 83: 220-230.
- [17] Liou HL et al. NPC2, the protein deficient in Niemann-Pick C2 Disease, consists of multiple glycoforms that bind a variety of sterols. *J. Biol. Chem*. 2006; 281 (48): 36710-36723.
- [18] Vanier MT. and Millat G. Structure and function of the NPC2 protein. *Biochimica et Biophysica Acta*. 2004; 1685: 14- 21.
- [19] Millat, Gilles et al. Niemann-Pick Disease Type C: Spectrum of HE1 Mutations and Genotype/Phenotype Correlations in the NPC2 Group. *AM. J. Hum. Genet*. 2001; 69: 1013-1021.
- [20] Klünemann HH et al. Frontal Lobe Atrophy Due to a Mutation in the Cholesterol Binding Protein HE1/NPC2. *Ann Neurol*. 2002; 52: 743-749.
- [21] Park WD et al. Identification of 58 Novel Mutations in Niemann-Pick Disease Type C: Correlation With Biochemical Phenotype and Importance of PTC1-Like Domains in NPC1. *Human Mutation*. 2003; 22: 313-325.
- [22] Vanier MT and Gilles Millat. Structure and function of the NPC2 protein. *Biochimica et Biophysica Acta*. 2004; 1685: 14-21.
- [23] Verot L et al. Niemann-Pick C disease: functional characterization of three NPC2 mutations and clinical and molecular update on patients with NPC2. *Clin Genet*. 2007; 71: 320-330.
- [24] Clark NE and Garman SC. The 1.9 Å Structure of human α -N-Acetylgalactosaminidase: the molecular basis of Schindler and Kanzaki diseases. *J. Mol. Biol*. 2009; 393: 435–447.
- [25] Gimpl, Gerald and Katja Gehrig-Burger. Cholesterol Reporter Molecules. *Biosci Rep*. 2007; 27: 335–358.
- [26] Schroeder F et al. Interaction of fluorescent delta 5,7,9(11),22-ergostatetraen-3 beta-ol with sterol carrier protein-2. *J. Biol. Chem*. 1990; 265: 151-157.

- [27] Smutzer, Greg, Brian F. Crawford, Philip L. Yeagle. Physical properties of the fluorescent sterol probe dehydroergosterol. *Biochimica et Biophysica Acta*. 1986; 862: 361-371.
- [28] Schroeder, Friedhelm et al. A Fluorescence Study of Dehydroergosterol in phosphatidylcholine Bilayer Vesicles. *Biochemistry*. 1987; 26: 2441-2448.
- [29] McIntosh AL. Fluorescence Techniques Using Dehydroergosterol to Study Cholesterol Trafficking. *Lipids*. 2008 December ; 43(12): 1185–1208.
- [30] Kwon, Hyock Joo et al. Structure of N-Terminal Domain of NPC1 Reveals Distinct Subdomains for Binding and Transfer of Cholesterol. *Cell*. 2009; 137 :1213-1224.
- [31] Moe PC and Heuck AP. Phospholipid hydrolysis caused by *Clostridium perfringens* α -Toxin facilitates the targeting of perfringolysin O to membrane bilayers. *Biochemistry*. 2010; 49: 9498-9507.